FINAL REPORT
Of research conducted under OTKA 100638 grant
Transcriptional regulation of the human ABCC6 gene, a cardiovascular risk factor
01.01. 2012.-31.12.2012

During this period we have published 5 papers and 3 further manuscripts are under revision.

The PI obtained a Marie Curie grant as an invited professor for the period of 2012-2014. During this period the project was managed first by Laszlo Buday than by Andras Varadi. The experienced postdoctoral fellow, Caroline Bacquet who started to work on the project left after 2 years. We could replace her only for the last year, with a less experienced researcher in the field.

Recessive loss-of-function mutations of the *ABCC6* gene lead to the development of pseudoxanthoma elasticum (PXE). PXE is characterized by elastic tissue fragmentation and calcification. The mechanisms of these biochemical alterations are still unknown but they cause dermatologic, cardiovascular and ophthalmologic symptoms. This is intriguing since the gene is essentially expressed in liver and not in the tissues, which are targeted by the disease. We have recently demonstrated that carriers of one allele with a loss-of-function mutation have an increased risk to develop coronary artery disease. Our research focused therefore on the transcriptional regulation of the *ABCC6* gene and the regulation of the transcription factors implicated in this process. Based on our results we went further and started to investigate the local regulatory changes at the *ABCC6* region and global genome-wide reactions to acute metabolic stress. In order to perform our experiments we needed extensive methodological improvements.

1/ Methodological improvement

Two major changes were realized during the last years. First, local analyses were substituted by genome-wide analyses; second, we switched from the previously used cell line models to a mouse model in several experiments.

We have successfully set up Western blot analysis for histone modifications after acidic protein extraction. We have improved our chromatin immunoprecipitation (ChIP) technique and set up ChIP-seq experiments in collaboration with Balint L Balint, Debrecen University. For the analysis of ChIP experiments we enlarged the panel of target genes tested and several new controls were systematically included. These technological improvements allowed us to perform successfully experiments on model cell lines and freshly prepared mouse hepatocytes.

We have also developed in collaboration with Pal Szabo (RCNS, HAS) the global quantification of DNA methylation and DNA hydroxymethylation by mass spectrometry. During the two years I spent in France, I learnt the use of Illumina 450k methylation platform allowing the analysis of almost 0.5 million CpGs separately. However, this technique is specific to human. Our further great achievement was therefore the setting up of DNA methylome sequencing, called reduced representation bisulfite sequencing (RRBS). This method allows the analysis of DNA methylation of approximately 1 million CpGs mainly in promoters and CpG islands.

The bioinformatic analysis of the obtained data was set up in the laboratory and is being performed in our research group. Currently we are developing a pipeline for the RRBS analysis.

2/ Transcriptional Regulation of ABCC6 (1 published manuscript)

In the course of the previous years our group elucidated which transcription factors (TF) regulate the hepatocyte-specific expression of the *ABCC6* gene. By using several complementary techniques we have demonstrated that C/EBPbeta acts as a primate-specific transcription factor in the 1st intron of the gene, C/EBPalpha bind the promoter and HNF4 alpha (HNF4α) recognizes an evolutionarily highly conserved binding site in the promoter. This latter TF is responsible for the regulation of tissue-specific expression of *ABCC6* and orchestrates the TF network. In addition, we have shown that the activation of different signal transduction cascades (PKC, MAPK, AMPK) inhibits the expression of the *ABCC6* gene *via* the inhibition of HNF4α.

3/ ERK1/2 phosphorylation of HNF4α (manuscript in preparation)

We have shown by ChIP-qPCR assays on HepG2 cells treated by phorbol ester (PMA) or epidermal growth factor (EGF) that HNF4α binding to the *ABCC6* proximal promoter decreases.

These experiments revealed several HNF4α binding sites in the *ABCC6* gene in addition the one already identified in the promoter. We could also demonstrate that ERK1/2 activation decreases HNF4α DNA binding already after 30 minutes and this effect is maintained until 24 hours. We confirmed these data by further ChIP-qPCR in several independent experiments. We investigated 8 well-known HNF4α target gene sequences (ABCC6 and 7 others) including a negative control region as well selected upon our ChIP-seq analyses. Our ChIP-seq experiments performed in collaboration with Balint L Balint showed similar results. We observed a considerable decrease (75%) in the genomic HNF4α binding sites and almost 50% after 30 minutes.

In addition, we investigated by immunofluorescence microscopy the effect of EGF and PMA on the nuclear localization of HNF4α in HepG2 cells. We observed that the TF is localized to the nucleus and the immunostaining slightly decreases already after 30 minutes and disappears after 24 hours. However no change was observed in the localization of the signal, suggesting that the HNF4α DNA binding decrease due to the activation of the kinases was not the consequence of a relocalisation but the loss of expression of the TF.

Next, in a different set of experiments we also investigated *in vitro* the phosphorylation of HNF4α by ERK1. The phosphorylation of HNF4α was determined by mass spectrometry. These experiments confirmed our hypothesis and showed that ERK1 is able to phosphorylate HNF4α at several distinct sites. We observed that ERK1 can efficiently phosphorylate at AMPK, PKA and p38 and other previously reported sites and we also detected a new previously not identified phosphorylation site on the C-terminal of the HNF4α protein. ERK1 didn't phosphorylate HNF4α at the serine targeted by PKC.

We conducted further experiments to demonstrate that the phoshorylation of HNF4α by ERK1/2 leads to functional alterations and explain the loss of DNA binding observed in ChIP experiments. We generated 9 HNF4α constructs harboring phosphomimetic mutants at sites either identified in our experiments or described previously in the literature. We have tested

these mutants in HeLa cells, which do not express the endogenous HNF4α. We analyzed the capacity of the constructs to transactivate luciferase constructs containing the *ABCC6* promoter. We have deciphered which phosphorylation sites of the protein has functional importance after the activation of the ERK1/2 pathway. Our data confirmed that the PKC phosphorylation site used as a control (not targeted by ERK1) abolished the transactivation capacity of the TF. The other sites tested were phosphorylated according to our data. Neither the PKA site (according to the literature), nor the new ERK1/2 sites had an effect on the HNF4α activity. Similarly, and in contrast to the previously published data the p38 phosphomimetic mutant had no effect on HNF4α activity. The only ERK1/2 target site with functional activity coincides with the AMPK target site. Phosphomimetic mutant of this site had a significantly decreased activity in our luciferase assays.

Finally, we have also performed ChIP-seq experiments on EGF activated HepG2 cells for histone H3K27 acetylation and H3K4 trimethylation. H3K4 trimethylation was observed in the promoter, while H3K27ac at two intragenic sites. We also observed that while global H3K27 acetylation levels were significantly decreased upon ERK1/2 treatment, H3K4 trimethylation did not importantly change.

The manuscript summarizing these data is in preparation.

4/ Epigenetic changes upon acute metabolic stress

As we have shown, HNF4α and C/EBPalpha and beta in human cells of hepatic and intestinal origin regulate ABCC6. Furthermore, it has been recently published that ABCC6 participates in ATP extrusion from hepatocytes toward the bloodstream. Our data also indicated that activation of AMPK (by increased intracellular AMP:ATP ratio) decreased ABCC6 expression. Altogether these data suggest that metabolic stress participates in the regulation of ABCC6 expression.

We have therefore set up a widespread mouse model on C57Bl/6 mice, generally used in these experiments. The model is called “fast-refed”, consisting of an overnight fasting (16h) followed by a 2 hours refeeding with normal chow. This model allows the analysis of the reaction of hepatocytes to acute metabolic stress by investigating non-treated, fasted and fast-refed animals.

Using this system we can monitor the DNA methylation, histone modifications and TF binding changes at the *ABCC6* regulatory regions. Our preliminary data indicate the H3K4 trimethylation and H3K36 trimethylation of the gene body in the hepatocytes of control animals. The data also suggest epigenetic changes upon fasting and refeeding. We also performed DNA methylation analysis on the same samples and RNA will also be analyzed for expression changes.

We decided to use global approaches (ChIP-seq, RRBS and RNA-seq) to monitor epigenomic changes due to the metabolic stress. Our samples are currently being processed for next generation sequencing.

3/ The effect of activation of ERK1/2 on ABCG2 transporters (1 published manuscript)

In parallel to these studies we investigated the effect of ERK1/2 on the different promoters of ABCG2 gene and our results were published in BBRC. In this manuscript we published the following findings:

HGF increases ABCG2 protein amount on the plasma membrane in HepG2 cells; HGF and PMA activate ABCG2 gene expression via both the A and B promoters; HGF activates ABCG2 expression via the ERK1/2 cascade mainly through the A promoter; We also described that oxidative stress induces ABCG2 without specificity for the variants and aryl hydrocarbon receptor (AhR) activates the expression of ABCG2 specifically via the B variant in HepG2 cells.

4/ Organization of scientific meetings and publication of invited reviews (2 published papers)

In 2013 and 2015 we organized the biannual international PXE meeting in Budapest, in our institute. Approximately 40 European and American participants were present in both meetings. At these meetings clinicians, geneticists, patients' advocacy group representatives and basic science researchers form a translational network, which is leading now to the starting of the first allele-specific clinical trial on PXE based on the results obtained in our group in Budapest.

In 2013 we wrote an invited review, which summarized the current biochemical knowledge on the ABCC6 protein, structural features, mutation patterns and potential allele-specific therapies by 4-phenyl butyrate (PBA), which functions as a chemical chaperone. We demonstrated in vivo for several mutations that (in KO mice expressing the mutant human protein) the human protein is mislocalized but upon PBA treatment the protein's localization becomes normal and rescues the phenotype. PBA's action is probably indirect since it is a histone deacetylase inhibitor (HDACi) and alters the expression of a lot of genes.

In the same publication we gave a detailed review on the transcriptional regulation of the gene and described the hypothesis that the ABCC6 gene is under metabolic regulation. We also proposed that phenotypic variability of the disease might be partially due to metabolic differences between patients.

In 2015 we wrote another review summarizing the recent advances in the molecular mechanisms of PXE and different hereditary calcification disorders having very similar symptoms. We also presented the animal models and the different potential therapeutical approaches to envisage.

We have also initiated and organized the 1st Hungarian Epigenetic Meeting in 2012 and than the 1st Danube Conference on Epigenetics in 2014 and we are currently organizing the 2nd Danube Conference on Epigenetics. These meetings were organized in collaboration with Balint L Balint with the help of the Hungarian Biochemical Society. We have an international scientific board. The conferences had approximately 150 participants including the 20 invited speakers from Europe. A great number of invited speakers are EMBO members and/or ERC grantee. More than half of the participants are non-Hungarian Europeans.

5/ Collaborative research projects (1 published paper)

a/ Thanks to the methodological improvements described in 1/ we were invited to participate in a FP7 Marie-Curie Research Training Network as an associated partner. The PI became the joint PhD supervisor of a student investigating the molecular alterations in Tourette syndrome. Tourette syndrome is characterized persistent tics. We apply RRBS to identify epigenetic changes in rat striatum in a model for Tourette syndrome. In these experiments we demonstrated general hypomethylation in lesioned striatum relative to the contralateral

control striatum. We observed both hypo and hypermethylation but hypomethylation was much more pronounced. We also observed that the detected changes are occurring mainly in intragenic regions, which are poor in CpG. However approximately 200 genes were targeted according to our data, the bioinformatic analysis is still ongoing.
A review on the potentials of epigenetic studies in the field was submitted, OTKA was acknowledged.

b/ The PI had the opportunity to spend two years as an invited professor (in the frame of a Marie Curie project) from 2012-2014 in France. During this period he worked in Genethon, the most important gene therapy institute in Europe. He studied the global DNA methylation alterations due to transfection of hematopoietic stem cells with lentiviral vector. In order to describe the cellular reaction he used the Illumina 450k system and developed a new bioinformatics tool for the analysis. The results of these experiments showed that genome-wide methylation alterations occur due to the transfection, mainly hypermethylation in CpG islands. The data also showed that the observed methylation changes didn't depend on genomic integration of the vector but much more on the viral envelope and the purification method used during the vector preparation. Interestingly, the CpGs undergoing hypermethylation in different vector preparations were highly overlapping and concerned approximately 1000 genes. The manuscript summarizing the data is under 2nd revision, OTKA is acknowledged in the text.

c/ As a collaborator, the PI also participated in the characterization of hypertrophic response of human cardiomyocytes obtained embryonic stem cells (hESC) and human induced pluripotent stem cell lines (hiPSC). The results showed that the different models are not identical and hiPSCs cannot be considered equivalent between each other and hESCs. The results were published in Stem Cell Reports, OTKA was acknowledged.

d/ The collaboration, which led to the development of DNA methylation and hydroxymethylation analysis by mass spectrometry and to the initial publication of a paper in the beginning of 2012 (the PI's previous OTKA grant was acknowledged) was continued between three groups of the RCNS, HAS (Pal Szabo and David Szuts and ours). We have demonstrated that the inhibition of DNA methylation by the DNA methyltransferase inhibitor 5-aza-deoxycytidine (AdC) induced DNA hydroxymethylation in some cell types while not in others. Our results show that this phenomenon is characteristic of hematopoietic cell lines.